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# Protective role for TLR4 signaling in atherosclerosis progression as revealed by infection with a common oral pathogen[1]

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## Abstract

**Background—**Clinical and epidemiological studies have implicated chronic infections in the development of atherosclerosis. It has been proposed that common mechanisms of signaling via toll like receptors (TLRs) link stimulation by multiple pathogens to atherosclerosis. However, how pathogen specific stimulation of TLR4 contributes to atherosclerosis progression remains poorly understood.

**Methods and Results—**Atherosclerosis-prone apolipoprotein-E null ($ApoE^{-/-}$) and TLR4 deficient ($ApoE^{-/-}TLR4^{-/-}$) mice were orally infected with the periodontal pathogen, *Porphyromonas gingivalis*. $ApoE^{-/-}TLR4^{-/-}$ mice were markedly more susceptible to atherosclerosis following oral infection with *P. gingivalis*. Using live animal imaging, we demonstrate that enhanced lesion progression occurs progressively and was increasingly evident with advancing age. Immunohistochemical analysis of lesions from $ApoE^{-/-}TLR4^{-/-}$ mice revealed an increased inflammatory cell infiltrate composed primarily of macrophages and IL-17 effector T cells (Th17), a subset linked with chronic inflammation. Furthermore, enhanced atherosclerosis in TLR4-deficient mice was associated with impaired development of T helper type-1 (Th1) immunity and regulatory T cell (Treg) infiltration. *In vitro* studies suggest that the mechanism of TLR4-mediated protective immunity may be orchestrated by dendritic cell interleukin (IL)-12 and IL-10, prototypic Th1 and Treg polarizing cytokines.

**Conclusions—**We demonstrate an atheroprotective role for TLR4 in response to infection with the oral pathogen, *P. gingivalis*. Our results point to a role for pathogen-specific TLR signaling in chronic inflammation and atherosclerosis.

### Keywords

Inflammation; Transgenic/Knockout Mice; Bacterial Infection; Monocytes/Macrophages

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**Disclosures**
None

## Introduction

The identification of atherosclerosis as a chronic inflammatory disease has emphasized the fundamental role of the immune system in disease pathogenesis (1). Detection of endogenous and microbial ligands by immune competent cells occurs via germ-line encoded pattern recognition receptors including the innate immune TLRs (2). Engagement via TLRs initiates acute inflammatory responses that are critical in host defense (3). Resident cells in human atherosclerotic plaque express TLRs (4). Animal studies employing hyperlipidemic mice have shown that TLR2, TLR4, and the downstream signaling molecule, MyD88, play an important role in diet-induced atherosclerosis (5-9). Reduced atherosclerotic development has been observed in TLR4-deficient ApoE$^{-/-}$ mice on high fat Western diet (5, 6, 8). We have previously shown that TLR2 also plays an important role in atherosclerotic progression independent of dietary lipids in hyperlipidemic ApoE$^{-/-}$ mice (7).

A subset of Gram-negative mucosal pathogens including *Chlamydia pneumoniae* and *Porphyromonas gingivalis* induce chronic and systemic inflammatory responses associated with atherosclerosis through TLR signaling (10-12). *P. gingivalis* induces a local inflammatory response that results in oral bone destruction manifested as periodontal disease, an inflammatory disease that affects 100 million people in the U.S (13). In addition to chronic inflammation at the initial site of infection, mounting evidence supports a role for *P. gingivalis*-mediated periodontal disease as a risk factor for systemic diseases, including atherosclerotic cardiovascular disease as well as diabetes and pre-term birth (10, 11, 14-18). *P. gingivalis* has been detected in human atherosclerotic plaque (19, 20) and animal models of *P. gingivalis* infection have validated human studies (21-26).

*P. gingivalis* induces pro inflammatory responses primarily through fimbriae-mediated signaling via TLR2 that is MyD88-dependent (27). In support of this, we previously demonstrated that a *P. gingivalis* fimbriae mutant, *fimA*, failed to accelerate atherosclerosis in ApoE$^{-/-}$ mice (22). Furthermore, we established that TLR2 signaling contributes in part to *P. gingivalis*-induced atherosclerosis in mice on a normal chow diet (28). In addition to signaling for proinflammatory responses via TLR2, *P. gingivalis* has developed mechanisms to evade detection and eradication by the immune system (29, 30). One such mechanism is modification of its lipid A, the biological core of bacterial LPS, universally recognized by the TLR4-MD2 complex (31). In response to environmental stimuli (32) and availability of the essential nutrient heme (33), *P. gingivalis* utilizes enzymes to modify the acylation and phosphorylation of its lipid A, resulting in differential recognition by the TLR4 complex (29, 30). In the present study, we demonstrate the unique ability of *P. gingivalis* to evade TLR4 signaling while inducing TLR2 dependent pro-inflammatory responses, reveals a protective role for TLR4 in chronic inflammatory atherosclerosis.

## Materials and Methods

### Mice

Male ApoE$^{-/-}$ and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). TLR4$^{-/-}$ mice on C57BL/6 background were provided by S. Akira (Osaka University). ApoE$^{-/-}$TLR4$^{-/-}$ mice were generated in our laboratory. Mouse genotypes were confirmed by PCR and experimental mice were age-matched. Mice were maintained under specific pathogen-free conditions and cared for in accordance with Boston University Institutional Animal Care and Use Committee.

### Bacteria

*P. gingivalis* strain 381 was grown anaerobically on blood agar plates (BBL; Becton Dickinson Co.) and used to seed-innoculate brain heart infusion broth (pH 7.4; BBL) supplemented with yeast extract (BBL), hemin (10μg/ml; Sigma), and menadione (1μg/ml). Colony forming units were standardized at an optical density at 660 nm of 1 (equivalent to 1 × $10^9$ CFU/ml) by spectrometry (ThermoSpectronic Genesys 20). LPS from *P. gingivalis* 381 was isolated using a modified Tri-Reagent protocol (29).

### Oral infection

Three independent experiments were performed with $ApoE^{-/-}$ (Total n=40) and $ApoE^{-/-}TLR4^{-/-}$ (Total n=30) mice and data were pooled. Mice were fed a normal chow diet (Harlan Teklad, Madison WI; Global 2018). Six-week-old male mice were given antibiotics (Sulfatrim) *ad libidum* in the drinking water for ten days, followed by a two-day antibiotic-free period. 100 μl of *P. gingivalis* 381 ($1\times10^9$CFU) suspended in vehicle (2% carboxymethylcellulose in PBS) was topically applied to the buccal surface of the maxillary gingiva five times a week for three weeks (34). Control mice received 100 ul of vehicle. Topical application of P. gingivalis to the buccal surface of the maxillary gingiva five times a week for three weeks induces alveolar bone loss in ApoE−/−mice (28). Mice were euthanized 13 wks after the final oral challenge (24wks of age). This time point is consistent with the time frame used in our prior studies (7, 28, 35).

### Magnetic Resonance Angiography (MRA)

MRA of the innominate artery was performed with vertical-bore Bruker 11.7 T Avance spectrometer (Bruker; Billerica, MA) as described (35). Mice were anesthetized with 0.5-2% inhaled isoflurane, placed into a 30mm vertical probe (Micro 2.5) maintained at 23°C. Respiration was monitored using a monitoring and gating system (SA Instruments, Wahkesha, WI). The un-gated 3D gradient echo MRA was acquired with the parameters: Slab thickness = 1.5 cm, flip angle = 45°, repetition time (TR) = 20 ms, echo time (TE) = 2.2 ms, field of view (FOV) = 1.5 × 1.5x 1.5 cm, matrix = 128 × 128 × 128, in-plane, number of average (NEX) = 4. Total scan time was ~ 25 min. Image reconstruction and analysis were performed using Paravision™. The 3D reconstruction of the MRA images was achieved by maximum intensity projection (MIP). The cross sections were chosen at 0.3-0.5mm distance below the subclavian bifurcation. Lumen area was manually defined and calculated with Image J (NIH) by 2 independent observers. Measurement reproducibility had an interclass correlation coefficient of 0.92.

### Atherosclerotic plaque assessment

Aortas were harvested and stained with Sudan IV as described (22). Digital micrographs were taken, and total area of atherosclerotic plaque was determined using IPLabs (Scanalytics, Inc) by a blinded observer.

### Immunohistochemistry

Mice were euthanized (n=4/group), perfused with 4% paraformaldehyde, and aortic arch with heart tissue was embedded in OCT freezing compound. Five μm serial cryosections were collected every 50 μm in the innominate artery and aortic sinus. In the innominate artery, cryosections were obtained from the region corresponding to the greatest plaque size as revealed by MRA, approximately 0.3 mm below the bifurcation of the innominate and subclavian arteries as described (35). Immunohistochemistry was performed using rat anti-mouse F4/80 (Serotec #MCA497R, Oxford, UK), rat anti-mouse CD4 (BD Biosciences # 550278 and Caltag Laboratories), rat anti-mouse CD8 (BD Biosciences #550281), rat anti mouse TLR2 (eBioscience # 13-9021-80) anti-mouse FOXP3 (Enzo Clone MF333F), rat

anti-mouse IL-17 (R&D Systems #MAB2276), or isotype controls (Serotec #MCA1125, Oxford, UK). Biotinylated anti-rat (mouse absorbed) IgG was used as secondary antibody (Vector Laboratories, Inc, Burlingame, CA). Digital micrographs were captured. Nuclei were counterstained with hematoxylin and positive cells were enumerated by microscopy. $F4/80^+$, $CD8^+$ T cells, $Foxp3^+$ T cells, For $IL\text{-}17^+$ each from three sections of four the quantitation T cells wereofcounted positive from were staining area, images acquired mice/ group. using an Olympus BX41 microscope at 100x magnification and automated color thresholding was performed using ImageJ (NIH).

### Flow cytometry

Anti-mouse antibodies included CD3 (BD Biosciences #553062), CD4 (BD Biosciences #553049, CD8 (BD Biosciences #553034), Ly6G, CD45 (BD Biosciences #550994) and isotype controls (BD Pharmingen) and IFN-γ (eBioscience #48-7311), IL-17A (eBioscience #51-7177), F4/80 (eBioscience #12-4801), and TLR2 (eBioscience #12-9022). Intracellular cytokine staining was performed using a mouse kit (BD Pharmingen # 559311). Samples were acquired on a BD LSR II flow cytometer (Becton Dickinson) and data were analyzed using FlowJo software (TreeStar, Inc.).

### Cell culture

For isolation of bone marrow derived dendritic cells (DCs), bone marrow cells were cultured in RPMI 1640 containing 10% FBS, 1x non-essential amino acids (MP Biomedicals), 50μM beta-mercaptoethanol (Gibco), 100 μg/ml streptomycin/100 IU penicillin (Cellgro), and 20 ng/ml recombinant mouse GM-CSF (Peprotech, Inc.) for 11 days. DCs were greater than 95% positive for CD11c. Non-adherent DCs were collected and re-plated in 24-well dishes at $2\times10^5$/cells/well in complete media without antibiotics before addition of *P. gingvalis* at MOI 25 and 50. After 24 hours, culture supernatants were collected, samples were clarified by centrifugation, and stored at −80 °C for ELISA.

### ELISA

Concentrations of IL-6, IL-12p40, and IL-10 in cell culture supernatants were determined by ELISA (BD OptEIA). Plasma was collected from a subset of experimental mice 16 wks post-infection and assayed by ELISA for *P. gingivalis*-specific antibody isotypes IgG1, IgG2b, IgG2c, and IgG3 as described (36) as follows. Bacteria were washed three times in PBS and fixed overnight at 4°C in 4% paraformaldehyde. Fixed-bacteria were washed 5x in PBS and protein concentration estimated by bicinchoninic acid (BCA) protein assay. Immulon 4HXB plates were coated overnight at 4°C with 10 μg/ml *P. gingivalis* suspension in PBS containing 0.05% sodium azide. Serial dilutions of mouse serum were plated and determination of IgG isotypes was conducted using the C57BL/6 Clonotyping Kit (SouthernBiotech). Quantitation of IgG was determined using a standard curve. ELISAs were developed using 4-Methylumbelliferyl phosphate (MUP) and read on spectrofluorometer (BioTek Synergy HT).

### Splenocyte re-stimulation assay

Splenocytes ($2\times10^6$/ml) were collected and stimulated with *P. gingivalis* soluble antigens (10μg/ml) in the presence of 1μg/ml anti-mouse CD28 (eBioscience) for 4 hrs and 10 μg/ml Brefeldin A (eBioscience). Cells were harvested, stained with anti-mouse antibodies (BD Pharmingen): CD3, CD4, and CD8. Intracellular cytokine staining for IL-17A (eBioscience) and IFN-γ (eBioscience) was performed using BD Cytofix/Cytoperm kit.

### Statistics

Normality of data was determined by visually inspecting for bell-shaped curves. A Mann-Whitney U test was performed to compare two independent samples with Prism 5 software (GraphPad Software Inc., San Diego, CA) with an alpha equal to 0.05 as considered significant. Two-way ANOVA was performed for analysis of % plaque between genotypes and infections. A value of $p < 0.05$ was considered significant.

## Results

### TLR4 deficiency confers enhanced susceptibility to chronic and progressive atherosclerosis following infection with *P. gingivalis*

ApoE$^{-/-}$ and ApoE$^{-/-}$TLR4$^{-/-}$ mice were orally infected with $10^9$ CFU *P.gingivalis* strain 381. The predominant lipid A species expressed by *P. gingivalis* 381 grown under standard laboratory conditions in the presence of excess heme was tetra-acylated nonphosphorylated (m/z 1380), which is TLR4 inert and immunologically silent (data not shown) (32). The minor lipid A species produced by strain 381 was penta-acylated monophosphorylated (m/z 1690) and has been demonstrated to act as both a weak TLR4 agonist and antagonist (29).

Progression of atherosclerosis in the innominate artery of individual mice was examined *in vivo* by magnetic resonance angiography (MRA). The innominate artery exhibits a high degree of lesion progression and expresses features of human disease including vessel narrowing, perivascular inflammation, and plaque disruption (35). The luminal area of the innominate artery of *P. gingivalis*-infected ApoE$^{-/-}$ and ApoE$^{-/-}$TLR4$^{-/-}$ mice decreased between baseline and 12 weeks compared to uninfected ApoE$^{-/-}$ and ApoE$^{-/-}$TLR4$^{-/-}$ mice (Fig. 1A) illustrating vessel narrowing and disease progression in infected mice. In *P. gingivalis* infected ApoE$^{-/-}$ mice, luminal area remained unchanged between 12 and 16 weeks. However, *P. gingivalis* infected ApoE$^{-/-}$TLR4$^{-/-}$ mice exhibited a progressive decline in luminal area at 0, 12, and 16 weeks following the first oral infection, indicative of progressive atherosclerosis. Luminal area was significantly smaller in ApoE−/−TLR4−/− mice compared to ApoE−/− mice at 16 weeks (p=0.03).

Assessed by microscopy in post-mortem sections, the innominate artery of uninfected ApoE$^{-/-}$ and ApoE$^{-/-}$TLR4$^{-/-}$ mice appeared as tightly packed layers of smooth muscle cells with uniform distribution about the circumference of the artery with no apparent lipids and inflammatory cells (Fig. 1B: ApoE$^{-/-}$, upper left; ApoE$^{-/-}$TLR4$^{-/-}$, upper right). Brown staining indicates the presence of macrophages. Plaque formation in the innominate artery of *P. gingivalis* infected ApoE$^{-/-}$ mice was modest and superficial, appearing as fatty streaks at the intimal surface (Fig. 1B ; lower left). In *P. gingivalis* infected ApoE$^{-/-}$TLR4$^{-/-}$ mice, we observed a significant increase in arterial plaque, which accumulated within subendothelial layers and coincided with the infiltration of inflammatory cells, including macrophages (Fig. 1B; lower right). In contrast to fatty streaks in infected ApoE$^{-/-}$ mice, plaques in infected ApoE$^{-/-}$TLR4$^{-/-}$ mice protruded into the arterial lumen. Higher resolution reveals the structure of the intima, media and adventitia in the innominate arteries of each group (Fig. 1C). Corresponding sections from MRA analyses revealed an increase in plaque area within the innominate artery of infected ApoE$^{-/-}$TLR4$^{-/-}$ mice compared to infected ApoE$^{-/-}$ mice (Fig. 1D bar graph). No significant differences in plaque area between uninfected ApoE$^{-/-}$ and ApoE$^{-/-}$TLR4$^{-/-}$ mice were observed.

In the absence of infection, no differences in en face total aortic lesion area, assessed by lipid staining, were observed between uninfected ApoE$^{-/-}$ and ApoE$^{-/-}$TLR4$^{-/-}$ mice (Fig. 2A and 2B). Consistent with our previous studies (22), infected ApoE$^{-/-}$ mice developed significantly more plaque than uninfected ApoE$^{-/-}$ controls. Aortas from infected ApoE$^{-/-}$TLR4$^{-/-}$ mice also demonstrated significantly more plaque than their uninfected,

genotype-matched ApoE$^{-/-}$TLR4$^{-/-}$ controls; however, plaque area was significantly greater in infected ApoE$^{-/-}$TLR4$^{-/-}$ mice compared to infected ApoE$^{-/-}$ mice. While the increase in lesion area in infected ApoE$^{-/-}$ mice largely localized to the atherosclerosis prone regions in the aortic arch, lesions in infected ApoE$^{-/-}$TLR4$^{-/-}$ mice occurred in the proximal as well as the distal aorta.

### TLR4 deficiency is associated with increased macrophage infiltration and expression of TLR2 in aortic lesions from *P. gingivalis* infected mice

The increased atherosclerotic plaque observed in infected ApoE$^{-/-}$TLR4$^{-/-}$ mice was accompanied by a significantly increased accumulation of macrophages within the aortic sinus while macrophage accumulation was not significantly increased in infected ApoE$^{-/-}$ mice (Fig. 3A and 3C, left). In agreement with previous findings (22), *P. gingivalis* infection resulted in increased expression of TLR2 within the aortic sinus of infected ApoE$^{-/-}$ mice, as well as in ApoE$^{-/-}$TLR4$^{-/-}$ mice, in areas where macrophages were found (Fig. 3B and 3C, right). TLR2 expression was also significantly higher in infected ApoE$^{-/-}$TLR4$^{-/-}$ mice compared to ApoE$^{-/-}$ mice (Fig. 3C, right).

Greater plaque area and infiltration of macrophages into plaque in ApoE$^{-/-}$TLR4$^{-/-}$ mice cannot be attributed to differences in plasma cholesterol or triglycerides, as these were similar among all groups (Cholesterol, mean+SE: uninfected ApoE$^{-/-}$, 476+22; infected ApoE$^{-/-}$, 449+24; uninfected ApoE$^{-/-}$TLR4$^{-/-}$, 500+36; infected ApoE$^{-/-}$TLR4$^{-/-}$, 512+24 mg/dL. Triglycerides, mean+SE: uninfected ApoE$^{-/-}$, 237+21; infected ApoE$^{-/-}$, 245+18; uninfected ApoE$^{-/-}$TLR4$^{-/-}$, 225+24; infected ApoE$^{-/-}$TLR4$^{-/-}$, 206+22 mg/dL).

### TLR4 deficiency promotes Th17/Treg imbalance in atherosclerotic lesions following infection with *P. gingivalis*

In infected ApoE$^{-/-}$ mice, we observed no increase in CD8+ T cells, TCD4$^{+}$ T cells or IL-17$^{+}$ cells in the innominate artery, compared to uninfected ApoE$^{-/-}$ mice (Fig. 4A and 4C). Accumulation of CD4$^{+}$ and CD8$^{+}$ cells within the innominate artery of infected ApoE$^{-/-}$TLR4$^{-/-}$ mice was dramatically increased compared to infected ApoE$^{-/-}$ mice. The abundance of T cells was accompanied by increased numbers of IL-17 expressing cells and markedly diminished numbers of Foxp3$^{+}$ expressing regulatory T cells (Treg cells) (Fig. and 4C). The marked increase in CD4$^{+}$, CD8$^{+}$ and IL-17$^{+}$ cells and the diminution of FoxP3$^{+}$ 4B Treg cells in infected mice in the absence of TLR4 expression (ApoE$^{-/-}$TLR4$^{-/-}$ mice) reveal that in the presence of TLR4 expression, TLR4 may be protective following *P. gingivalis* infection that serves to prevent the infiltration of IL-17$^{+}$ T cells and enhance the numbers of Foxp3$^{+}$ Treg cells in the inflammatory lesion.

### IgG humoral immunity and Th1 responses are altered in the absence of TLR4

Infection with *P. gingivalis* induced a robust IgG1 response in both ApoE$^{-/-}$ and ApoE$^{-/-}$TLR4$^{-/-}$ mice, indicating preservation of IgG1-mediated humoral immunity in the absence of TLR4 (Fig. 5A). However, *P. gingivalis*-infected ApoE$^{-/-}$TLR4$^{-/-}$ mice produced significantly reduced IgG2b (Fig. 5B) and IgG3 (Fig. 5D) responses compared to ApoE$^{-/-}$ mice, IgG subclasses that are associated with Th1 responses (37). IgG2c levels were increased to a similar level in infected ApoE$^{-/-}$ and ApoE$^{-/-}$TLR4$^{-/-}$ mice (Fig. 5C).

We restimulated splenocytes from experimental mice with *P. gingivalis* soluble antigens and identified responsive cells that express the effector cytokines IFN-γ and IL-17. T cells from uninfected mice did not exhibit cytokine expression in response to *P. gingivalis* antigens. We observed a high percentage of IFN-γ expressing CD4$^{+}$ (Fig. 6A and 6C) and CD8+ (Fig. 6B and 6C) T cells from *P. gingivalis* infected ApoE$^{-/-}$ mice. In contrast, the majority of responsive CD4$^{+}$ (Fig. 6A and 6D) and CD8+ T cells (Fig. 6B and 6D) from infected

ApoE$^{-/-}$TLR4$^{-/-}$ mice expressed IL-17. A small subset of CD8+ (14%) T cells from infected ApoE$^{-/-}$TLR4$^{-/-}$ mice also expressed IFN-γ. Although the number of reactive T cells indicates that these responses may not be antigen specific, they were specific to *P. gingivalis* infection, as T cells from uninfected mice failed to respond to stimulation with antigens. These results suggest that in the absence of TLR4, *P. gingivalis* infection results in impaired Th1 immunity and IL-17 skewing.

### Th1 and Treg polarizing cytokine production following *P. gingivalis* infection is impaired in dendritic cells from TLR4 deficient mice

Activation of TLRs on dendritic cells (DC) triggers the release of cytokines that play decisive roles in modulating T helper subset differentiation from naïve CD4$^{+}$ cells (38). To investigate the role of *P. gingivalis*-induced TLR4 activation in DC production of T cell polarizing cytokines, DCs from wild type and TLR4$^{-/-}$ mice were infected with *P. gingivalis* and expression of T cell polarizing cytokines was examined. *P. gingivalis* induced the production of IL-12 (Fig. 7A), IL-10 (Fig. 7B), and IL-6 (Fig. 7C), in DCs from ApoE$^{-/-}$ mice. Production of these cytokines was markedly reduced in DCs from ApoE$^{-/-}$ TLR4$^{-/-}$ mice. These results suggest that TLR4 is necessary for production of these cytokines following *P. gingivalis* infection. The abrogated DC production of T cell polarizing cytokines in the absence of TLR4 may be responsible for impaired development of Th1/Treg effector immunity as well as the enhanced IL-17 expression in T cell populations within plaque of ApoE$^{-/-}$TLR4$^{-/-}$ mice.

## Discussion

Common chronic infections may contribute to up to 40% of newly developed atherosclerotic cases (39). A role for *P. gingivalis*-mediated periodontal disease as a risk factor for atherosclerotic cardiovascular disease is well documented (10, 11, 14-18). The observation that innate immune signaling triggered by *P. gingivalis* is dysregulated within atherosclerotic lesions has sparked interest in the association between oral infection and induction of innate immune cascades in atherosclerosis progression (40). Most experimental studies have focused on the proatherogenic consequence of TLR signaling in mouse models of atherogenesis; many involving the influence of high fat diet (5, 6, 8, 12). In contrast to studies reporting diminished high fat diet-induced atherosclerosis in TLR4 deficient mice, we report the unexpected finding that TLR4-deficient mice are markedly more susceptible to atherosclerosis following infection with *P. gingivalis*. Live animal imaging demonstrated that enhanced disease severity occurred progressively, long after cessation of the infectious stimulus and at two anatomically relevant sites, in large (aortic sinus) and medium (innominate artery) sized vessels. Enhanced atherosclerosis progression in ApoE$^{-/-}$TLR4$^{-/-}$ mice compared to ApoE$^{-/-}$ mice is unlikely to be due to differences in plasma cholesterol or triglycerides, which were similar among all groups. Minimal atherosclerotic lesion area in the innominate artery was observed in uninfected ApoE−/− mice and this is likely due to the fact that animals were fed a normal chow diet. In our recent study in which atherosclerosis progression was examined using MRA in the innominate artery of uninfected and P. gingivalis-infected ApoE−/− mice, animals were fed a high fat diet for the duration of the study (35). High fat diet enhances atherosclerosis progression in ApoE−/− mice. In the absence of high fat diet and infection, plaque accumulation within the aorta and innominate artery progresses more slowly and is minimal at the time point examined in the present study. Effective control of immune-mediated pathology in *P. gingivalis* infected ApoE$^{-/-}$mice coincided with an increase in Treg within the innominate artery. In contrast, the exacerbated inflammatory pathology in *P. gingivalis* infected ApoE$^{-/-}$TLR4$^{-/-}$ mice was associated with increased lesion macrophage numbers, T cell infiltration and enhanced expression of IL-17. Regulatory T cells (Tregs) play a critical role in maintaining

immunological tolerance and controlling the extent of immune-mediated pathology, especially in cases of chronic infection (41, 42). Our studies indicate that in the absence of TLR4, mice fail to develop protective Th1 immunity and were unable to regulate adaptive immune responses mediated by Th17 cells following *P. gingivalis* infection. We propose that this results in a breakdown of immunological tolerance, owing to impaired Treg function, leading to unrestricted activation of pathogenic T cells that mediate arterial inflammation. The unique TLR4 evasive properties of *P. gingivalis* lipid A position this organism to disrupt effector T cell mechanisms at the level of dendritic cell activation, the interface of innate and adaptive immunity.

TLR2 expression was increased markedly in aortic lesions by P. gingivalis infection in ApoE−/− mice and further increased in ApoE−/−TLR4−/− mice. It is plausible that enhanced TLR2 expression in ApoE−/−TLR4−/− mice may have contributed to increased vascular inflammation and atherosclerosis in ApoE−/−TLR4−/− mice. Thus, the increase in atherosclerosis in ApoE−/− TLR4−/− mice may be a result of not only TLR4 deficiency, but also high TLR2 expression. This increased TLR2 expression in activated macrophages and the endothelium may reflect the development and maintenance of a hyper-inflammatory state in the absence of TLR4 expression. This observation was an unexpected finding of this study, as was the finding that plaque development was enhanced in the absence of TLR4.

Our results also showed that following in vitro restimulation with antigen, T cells from P. gingivalis-infected ApoE−/−TLR4−/− mice predominantly produced IL-17, while IFN-gamma was the predominant cytokine produced by T cells from infected ApoE−/− mice. We also demonstrate that TLR4-deficiency was associated with markedly inhibited production of the Th1 polarizing cytokine, IL-12 by P. gingivalis-infected dendritic cells. Taken together, our findings suggest that an initially impaired Th1 response in TLR4-deficient mice results in Th17 skewing of the adaptive immune response, which may be the mechanism for exacerbated atherosclerosis. Indeed, research supports a complex relationship between the Th1 and Th17 cell lineages, and many T cells expressing IL-17 co-express IFN-gamma. We previously reported that IFN-gamma is significantly up-regulated at the protein level in atherosclerotic lesions from P. gingivalis-infected ApoE−/− mice relative to uninfected controls (28). This finding is in agreement with IFN-gamma contributing to the development of atherosclerosis as suggested by one study (43). Several studies demonstrate that both IFN-gamma and IL-17 may be pro-atherogenic in mouse models, and circulating levels of both of these cytokines are increased in patients with coronary atherosclerosis (44). A large body of literature also supports a pro-atherogenic role for IL-17 in mouse models of atherosclerosis, and neutralization of IL-17 has been demonstrated to reduce pathogen- and diet-induced atherosclerosis in ApoE−/− mice (45). These studies demonstrate that the development of atherosclerosis is multifactorial and may be influenced by the inciting stimulus (i.e., high fat diet vs. pathogen-mediated).

Notably, humans are specifically impaired in their ability to recognize penta-acylated lipid A, a phenomenon reflecting bacterial adaptation to the human host (46). Species-specific discrimination of atypical penta-acylated lipid A is mediated by a hypervariable 82-amino acid sequence in the middle region of TLR4, a region where human polymorphisms are common (31, 46). Our results provide a mechanistic link regarding the conflicting reports on the association of human TLR4 polymorphisms and atherosclerotic diseases. Thus, although we only see a hyper inflammatory phenotype in TLR4-deficient mice, it is plausible that common human TLR4 polymorphisms (47-49) that attenuate receptor signaling may predispose individuals to an increased risk of atherosclerosis associated with bacterial infection, which is in contrast to atherosclerosis risk associated with a Western high fat diet.

Recent studies reported that ApoE$^{-/-}$TLR4$^{-/-}$ mice fed a high fat diet and infected intranasally with *C. pneumoniae* exhibited diminished atherosclerosis compared to infected ApoE$^{-/-}$ mice (12). A separate report demonstrated a protective role for TLR4 deficiency in diet-induced atherogenesis (8). It is important to note that these results could not be recapitulated under germ-free conditions (50), indicating a potential interaction between hyperlipidemia and indigenous microbes. Based on these observations it was proposed that common mechanisms of signaling via TLR2, TLR4 and MyD88 link stimulation by multiple pathogens and endogenous ligands to atherosclerosis, and that therapeutic TLR4 antagonism could prove beneficial in the treatment of chronic atherosclerosis (8, 51, 52). Our results clearly point to a critical role for specific TLR signaling, in particular, TLR4, in chronic inflammation and atherosclerosis induced by *P. gingivalis*. Our results raise caution for the safety and efficacy of TLR4 antagonists for the treatment of atherosclerosis, especially in patients with co-morbid conditions including periodontal disease and other infectious diseases.

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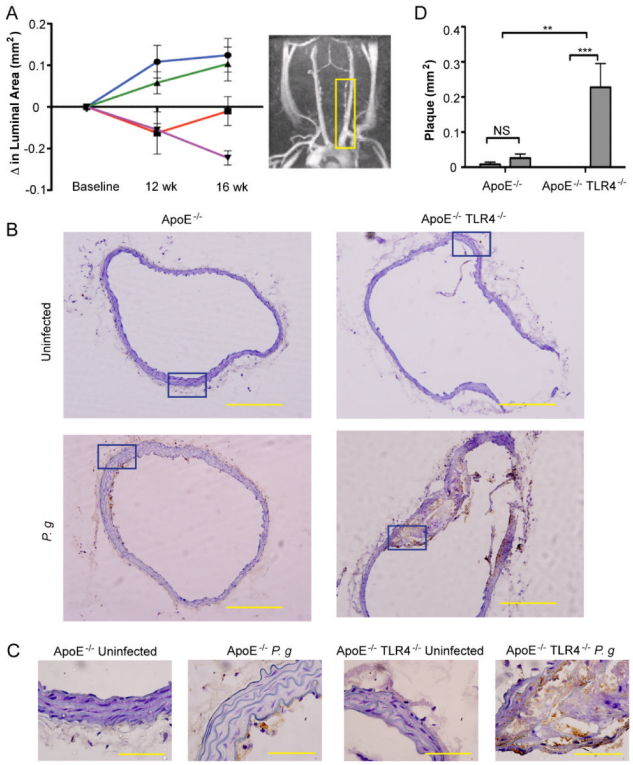


**Figure 1. TLR4 deficiency confers enhanced susceptibility to atherosclerosis in the innominate artery following infection with *P. gingivalis***

Innominate arteries were imaged by MRA at baseline (week 0), 12, and 16 wks following first oral infection. (A) The temporal change in luminal area ($mm^2$) was calculated for individual mice (n=5/group). Inset, representative MRA image indicating the innominate artery (yellow box), where measurements were taken. Uninfected $ApoE^{-/-}$ (blue); *P. gingivalis* infected $ApoE^{-/-}$ (red); Uninfected $ApoE^{-/-}$ $TLR4^{-/-}$ (green); *P. gingivalis* infected $ApoE^{-/-}TLR4^{-/-}$ (purple). (B) Representative hematoxylin staining from each group in innominate artery with F4/80 staining (macrophages stain brown). Scale=20μm. (C) Visualization of intima, media and adventitia of representative images. Area indicated in 1C (blue box). Scale=5μm. (D) Plaque area within the innominate artery measured from histological images using IPLab software (Becton, Dickinson and Company). (n=5/group). $^{**}p<0.01$, $^{***}p<0.001$. White bar: Uninfected. Gray bar: *P. gingivalis* infected.

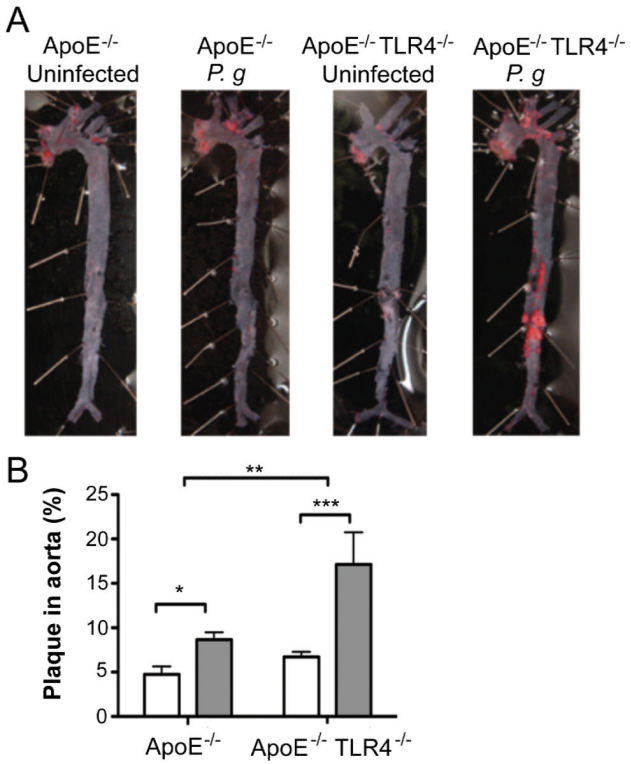


**Figure 2. TLR4 deficiency confers enhanced susceptibility to atherosclerosis in the aorta following infection with *P. gingivalis***

(A) Sudan IV staining of aorta *en face* lesions 16 wks following first infection with *P. gingivalis*. (B) Quantification of lipid content within the total aorta of uninfected (white bars) and *P. gingivalis* infected mice (gray bars) (n=10-13/group). Percentage of aorta occupied by lipids was calculated using IPLab software (Becton, Dickinson and Company). *$p$<0.05, ***$p$<0.001. **p< 0.01.

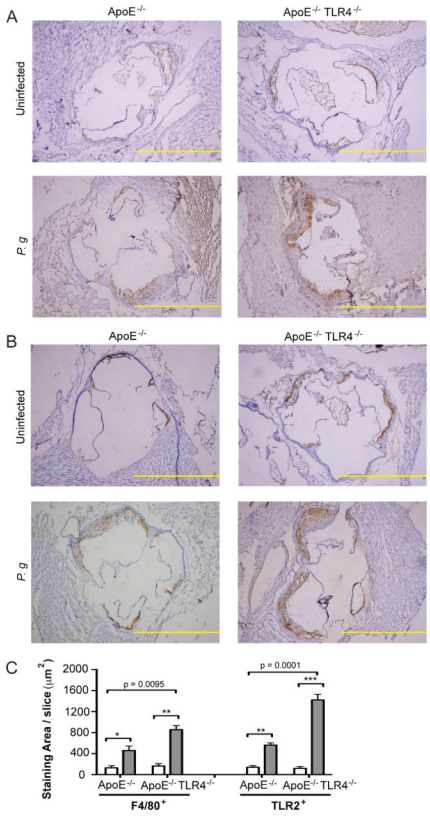


**Figure 3. TLR4 deficiency is associated with increased macrophage influx and TLR2 expression in atherosclerotic plaques following infection with *P. gingivalis***

Aortic sinus sections from uninfected and *P. gingivalis* infected ApoE$^{-/-}$ and ApoE$^{-/-}$TLR4$^{-/-}$ mice were stained for F4/80 and TLR2 positive cells with hematoxylin counterstaining. Representative aortic sinus sections stained for (A) F4/80 and (B) TLR2. Scale=100μm. (C) Quantification of positive staining area using ImageJ software (NIH). White bar: Uninfected. Gray bar: *P. gingivalis* infected. Three sections from n=4/group were analyzed. Data are mean ± SD positive staining area/slice. Intra-genotype comparisons were calculated by Mann Whitney U test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Inter-genotype comparisons were calculated by Two-way ANOVA, $p=0.0001$, $p=0.0095$.

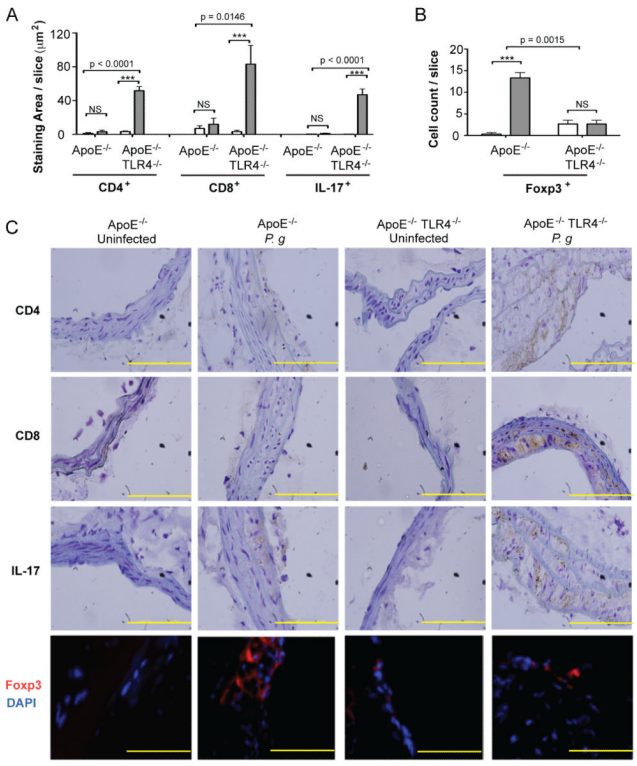


**Figure 4. TLR4 deficiency promotes Th17/Treg imbalance in atherosclerotic lesions following infection with *P. gingivalis***

Quantitative immunohistochemistry of (A) CD4, CD8, IL-17 positive staining area in the innominate artery of uninfected and *P. gingivalis* infected ApoE$^{-/-}$ and ApoE$^{-/-}$TLR4$^{-/-}$ mice using ImageJ software (NIH) or (B) Foxp3 positive cell count. Three sections from n=4 mice/group were analyzed. White bar: Uninfected. Gray bar: *P. gingivalis* infected. Data are mean ± SD of positive staining area/slice or cell count/slice. Intra-genotype comparisons were calculated by Mann Whitney U test ***$p$<0.0001. NS= not significant. Inter-genotype comparisons were calculated by Two-way ANOVA (indicated $p$ values). (C) Representative immunohistochemistry of CD4, CD8, IL-17, and Foxp3 positive cells in the innominate artery of uninfected and *P. gingivalis* infected ApoE$^{-/-}$ and ApoE$^{-/-}$TLR4$^{-/-}$ mice. Scale=5μm.

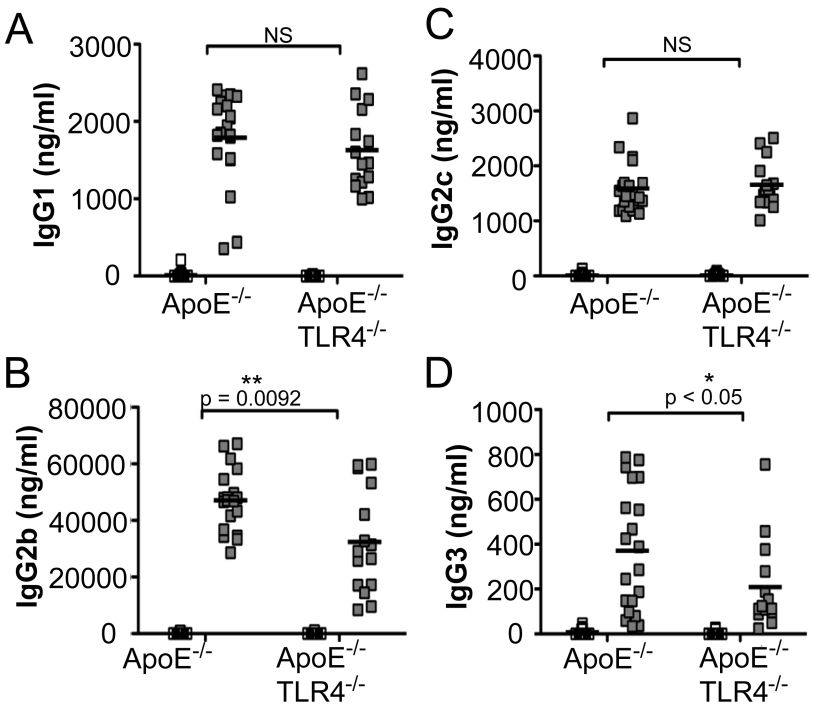


**Figure 5. *P. gingivalis*-specific antibody isotypes IgG1, IgG2b, IgG2c, and IgG3**

*P. gingivalis*-specific IgG production in uninfected ApoE$^{-/-}$ (n=15), *P. gingivalis* infected ApoE$^{-/-}$ (n=21), uninfected ApoE$^{-/-}$TLR4$^{-/-}$ (n=17) and *P. gingivalis* infected ApoE$^{-/-}$TLR4$^{-/-}$ (n=14) mice as measured by ELISA. (A) IgG1, (B) IgG2b, (C) IgG2C, (D) IgG3. White squares= uninfected, Gray squares= *P. gingivalis* infected. Data were analyzed by Student's t-test. *$p$<0.05, **$p$=0.0092. NS=not significant.

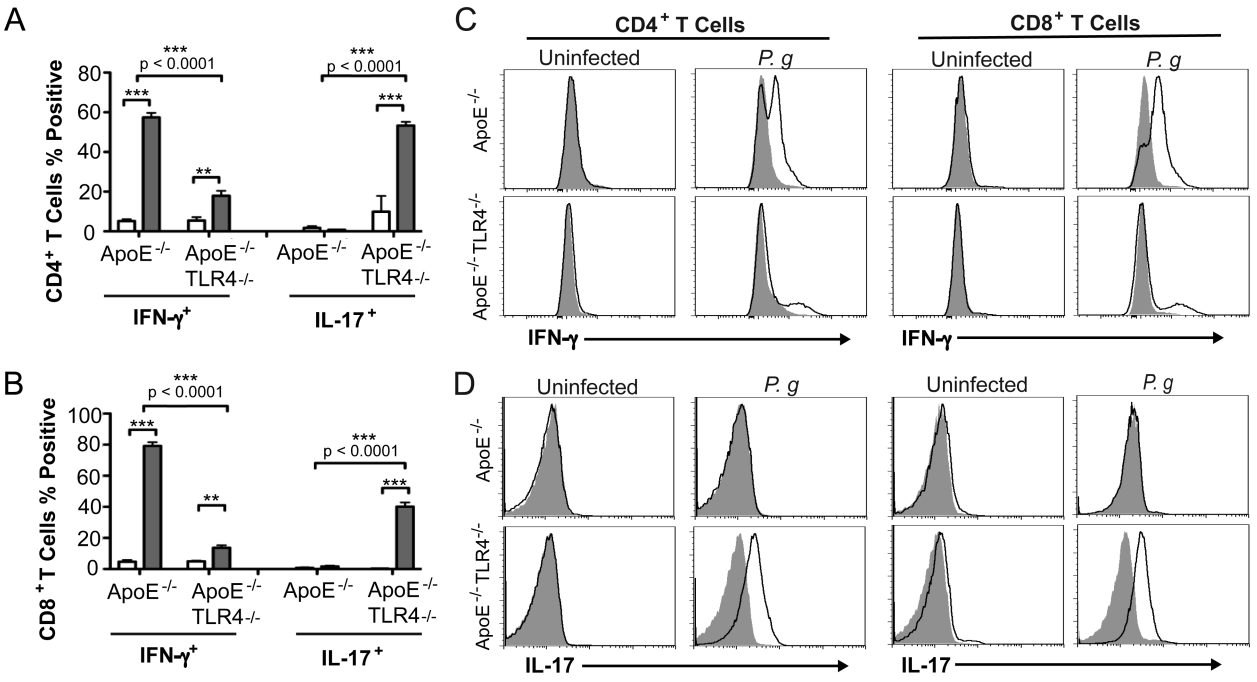


**Figure 6. Impaired Th1 immunity and Th17 skewing in *P. gingivalis* infected TLR4-deficient mice**

Expression of IFN-γ and IL-17 in (A) $CD4^+$ and (B) $CD8^+$ splenic T cells from uninfected and *P. gingivalis* infected mice following in vitro stimulation with 10 μg/ml P. gingivalis soluble antigens and 1 μg/ml anti-mouse CD28 for 4h in the continuous presence of Brefeldin A. Data represent mean ± SD, n=4/group. White bar= uninfected, Gray bar= *P. gingivalis* infected. Intra-genotype comparisons were calculated by Mann Whitney U test. $^{**}p<0.01$, $^{***}p<0.001$. Inter-genotype comparisons were calculated by Two-way ANOVA with Bonferonni's post-test. (C) Representative flow cytometry gating for the analysis of cytokine secreting $CD4^+$ and $CD8^+$ T cells.

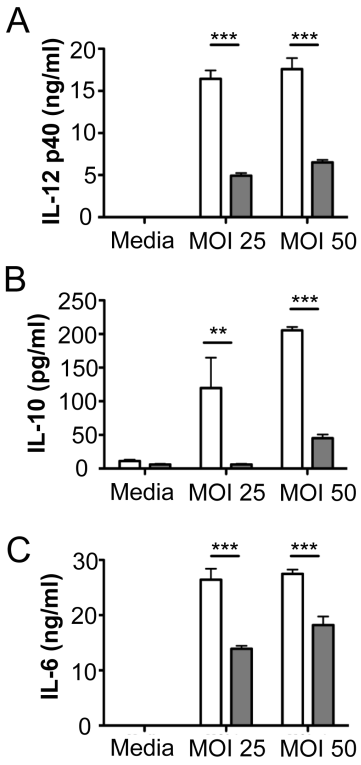


**Figure 7. Th1 and Treg polarizing cytokine production following *P. gingivalis* infection is impaired in dendritic cells from TLR4 deficient mice**

Bone marrow derived dendritic cells from C57BL/6 (white bars) and TLR4$^{-/-}$ (gray bars) mice were infected with P. gingivalis at the indicated multiplicity of infection (MOI). After 24h, supernatants were analyzed for (A) IL-12, (B) IL-10, and (C) IL-6 by ELISA. Bars represent mean ± SD from triplicate cultures. **$p$<0.01, ***$p$<0.001 by Student's t-test.